AD-A167 962 STRUCTURE OF ANTIBODIES BY X-RAY DIFFRACTION OF MONOMOLECULAR LAYERS AND B..(U) STANFORD UNIV CALIF STAUFFER LAB FOR PHYSICAL CHEMISTRY H M MCCONNELL

1/1

UNCLASSIFIED MAR 86 N00014-83-K-0349 F/G 6/1 NL

MICROCOPY CHART

(12)

OFFICE OF NAVAL RESEARCH

Contract N00014-83-K-0349

FINAL REPORT

March 1986

STRUCTURE OF ANTIBODIES BY X-RAY DIFFRACTION OF MONOMOLECULAR LAYERS AND BY NUCLEAR MAGNETIC RESONANCE

Harden M. McConnell
Stauffer Laboratory for Physical Chemistry
Stanford University
Stanford, CA 94305

FINAL REPORT FOR ONR CONTRACT N00014-83-K-0349

Structure of Antibodies by X-Ray Diffraction of Monomolecular Layers and by Nuclear Magnetic Resonance

Harden M. McConnell
Stauffer Laboratory for Physical Chemistry
Stanford University
Stanford, CA 94305

This contract research has been most successful, as the attached list of publications will indicate. The essential point is that we have demonstrated that a remarkably large amount of information about the combining site structure of a monoclonal anti-spin label (paramagnetic free radical) antibody can be determined from the NMR spectrum of this protein. This opens the way to the study of structural changes in the combining site due to somatic cell mutations (small numbers of amino acid substitutions). This work is now in progress. In the future, we hope to tailor make antibody combining sites having novel properties, such as enzymatic activity.

The equipment purchased under this grant has had a major, spectacular, favorable effect on the research in my laboratory. Briefly, the research objectives mentioned in the proposal have been achieved in a most satisfactory manner. In addition a number of related discoveries have been made. Finally, the equipment purchased under this grant has been of tremendous, critical value to other ongoing work in this laboratory. We believe the following list of publications, with titles, and abstracts, will provide an adequate report on our work.

The initial phases of this work were directed towards applying and developing the technique of surface X-ray diffraction for the study of antibody structure. During the course of the contract, there was a significant change, not in objective (antibody structure) but in our approach to this problem (NMR rather than X-ray diffraction). This change was brought about by two factors: (1) In addition to a number of difficult technical problems that were encountered, the beam time available to us per calendar year at the Stanford Synchrotron Radiation Laboratory (SSRL) proved to be too short to permit significant progress. (2) We found that the NMR spectra of a monoclonal anti-spin label antibody provide a remarkably powerful and much more rapid approach to this problem. Both lines of research are summarized below.

The surface X-ray diffraction studies were carried out at SSRL by Mr. Michael Seul, with the collaboration of Dr. Peter Eisenberger (Exxon). The principal achievements of our work include the design and construction of a He/H_2O vapor chamber that makes it possible to control the humidity over the sample, and at the same time to carry out surface reflection-diffraction. In addition, a second device was designed that permitted experiments to be carried out on hydrated monolayer samples contained between two thin mica sheets. This made transmission/diffraction experiments possible. Exploratory experiments were carried out with lipid monolayers (and multilayers), and with and without hapten-bound antibodies. A special iodine-containing lipid hapten was synthesized, to which monoclonal anti hapten antibodies were bound. Two approaches were considered: the first was an attempt to detect the presence of crystalline lipid antibodies by their effect in producing a superstructure in crystalline lipid monolayers, and the second was to detect directly the diffraction by the two-dimensional monolayer of crystalline antibodies. Simultaneously, experiments were initiated to observe this antibody crystallization by using freeze fracture electron microscopy and by measurements of lateral diffusion using fluorescence photobleaching experiments. Unfortunately, due to technical difficulties and the severe shortage of SSRL beam time, no significant diffraction data were collected. On the other hand, the proposed study does appear to be entirely feasible given proper conditions.

During the course of the X-ray diffraction studies, Dr. Jacob Anglister, Mr. Tom Frey, and I began a series of experiments on the proton nuclear magnetic resonance spectrum of a monoclonal anti spin label antibody. The spin label is paramagnetic; hence the difference spectrum, (NMR without spin label) - (NMR with spin label), reveals the proton signals of just those protons in the combining site region. It was also found that the hybridoma producing this monoclonal antibody could be adapted to grow on selectively deuterated amino acids. These developments, plus a technique to obtain distance information from the spectra, made it clear to us that NMR was the method of choice for quickly obtaining approximate information on antibody combining site structure. We have therefore focused on this promising and now proven approach.

Research publications directly related to the content of the proposal

286. "Magnetic resonance of a monoclonal anti-(spin-label) antibody," J. Anglister, T. Frey and H. M. McConnell. Biochemistry 23, 1138-1142 (1984).

290. "Distances of tyrosine residues from a spin label hapten in the combining site of a specific monoclonal antibody," J. Anglister, T. Frey and H. M. McConnell. Biochemistry 23(22), 5372-5375 (1984).

295. "Non-aromatic amino acids in the combining of a monoclonal anti spin label antibody," T. Frey, J. Anglister and H. M. McConnell. Biochemistry 23, 6470 -6473 (1984).

296. "Allogeneic stimulation of cytotoxic T cells by supported planar membranes," A. Brian and H. M. McConnell. Proc. Natl. Acad. Sci., USA, 81(19), 6159-6163 (1984).

298. "NMR technique to assess the contributions of the heavy and light chains to an antibody combining site," J. Anglister, T. Frey and H. M. McConnell, Nature, 315, 65-67 (1985).

305. "Diversity of molecular recognition: The combining sites of monoclonal anti-spin label antibodies", H. M. McConnell, T. Frey, J. Anglister, M. Whittaker, NATO meeting, Enrica sicily (1985).

Research publications benefitting directly from equipment grant, not included in the proposal

296. "Allogeneic stimulation of cytotoxic T cells by supported planar membranes", A. Brian & H. M. McConnell, Proc. Natl. Acad. Sci., USA, 81 (19), 6159-6163 (1984).

297. "Antigen presentation by supported planar membranes containing affinity purified I-A^d", T. Watts, A. A. Brian, J. W. Kappler, P. Marrack & H. M. McConnell, Proc. Natl. Acad. Sci. USA, 81, 7564-7568 (1984).

301. "Heterogeneity of phospholipid mobility in endothelium cells depends on cell substrate", M. Nakache, A. B. Schreiber, H. Gaub & H. M. McConnell, Nature, 317, 75-77 (1985).

302. "T cell activation by peptide antigen: effect of peptide sequence and method of antigen presentation", T. H. Watts, J. Gariepy, G. K. Schoolnik & H. M. McConnell, Proc. Natl. Acad. Sci., USA, 82, 5480-5484 (1985).

304. "Supported planar mambranes in studies of cell-cell recognition in the immune system", H. M. McConnell, T. H. Watts, R. M. Weis & A. A. Brian, Biochim Reviews, to be submitted.

306. "Topological distribution of surface markers on Endothelial cells", M. Nakache, H. E. Gaub, A. B. Schreiber, H. M. McConnell, Science.

310. "Total internal reflection fluorimetry on single T-lymphocytes in contact with supported lipid bilayers", H. E. Gaub, A. A. Brian, T. H. Watts & H. M. McConnell, Carnegie-Mellon University conference.

312. "T-cell mediated association of peptide antigen and MHC detected by energy transfer in an evanescent wave field", T. H. Watts, H. E. Gaub & H. M. McConnell, Nature, submitted.

Publications with Abstracts:

1. Magnetic Resonance of a Monoclonal Anti-(Spin-Label) Antibody, J. Anglister, T. Frey and H. M. McConnell, Biochemistry 23, 1138 (1984).

The nuclear magnetic resonance spectra of monoclonal Fab antibody fragments have been recorded in the absence and presence of a specific spin-label dinitrophenyl hapten. The difference spectra reveal the presence of 11-12 aromatic amino acids in the region of the combining site. By selective deuteration of this hybridoma antibody, these amino acids have been identified as three tryptophans, six or seven tyrosines, one phenylalanine, and one histidine. Difference spectra have also been recorded that depend on ring-current chemical shifts. The latter difference spectra show that among the 11-12 amino acids there are two tyrosines and one tryptophan in close proximity to the dinitrophenyl (INP) ring. On the basis of ultraviolet absorption measurements, it is concluded that this tryptophan is stacked against the DNP ring. Selective deuteration of hybridoma antibodies directed against a paramagnetic hapten provides a powerful new approach for the study of the structural basis of antibody diversity and specificity.

2. Distances of Tyrosine Residues from a Spin Label Hapten in the Combining Site of a Specific Monoclonal Antibody, J. Anglister, T. Frey and H. M. McConnell, Biochemistry, 23(22), 5372-5375 (1984).

The nuclear magnetic resonance spectra of a Fab fragment of a monoclonal antibody specifically directed against a nitroxide spin-label hapten have been recorded at different concentrations of the hapten. The hybridoma producing this antibody was grown on deuterated phenylalanine, tryptophan, and 3,5-dideuteriotyrosine or 2,6-dideuteriotyrosine. Difference spectra — without hapten minus with hapten — were calculated for each concentration of hapten. The difference spectra reveal five well-resolved singlet proton resonance signals from tyrosine deuterated in the 3,5-positions (H 2,6 Tyr) and nine from tyrosine deuterated in the 2,6-positions (H 3,5 Tyr). The measured intensities of these signals as a fu nction of combining site occupation have been interpreted in terms of a theory involving intrinsic line widths (T_2), the hapten off-rate (k), and distances to the paramagnetic center. Good agreement with theory is found for all of the isolated proton signals. The best estimate of k is 350 s^{-1}; distances in the range 13 to <q A are calculated. Extension of this analysis to other amino acids is discussed.

3. Non-Aromatic Amino Acids in the Combining of a Monoclonal Anti Spin Label Antibody. T. Frey, J. Anglister and H. M. McConnell, Biochemistry, 23, 6470-6473 (1984).

The nuclear magnetic resonance spectra of monoclonal Feb antibody fragments have been recorded in the absence and presence of a specific spin-label dinitrophenyl hapten. The difference spectra reveal the presence of about 50 amino acids in the region of the combining site. By selective deuteration and using double difference spectra, all the resonances in the spectral region -1 ppm to 1.5 ppm have been identified. We have found that in the combining site region there are 4 or 5 valines, certainly 3 and possibly 5 threonines, 3 or 4 leucines, 2 or 3 isoleucines, and 6 or 7 alanines. Selective deuteration of methionine and lysine reveals 1 methionine and 2 lysines in the difference spectra. All of these amino acids are estimated to be within 17A of the paramagnetic hapten. By using difference spectra involving low fractional occupancy of the combining site with the spin-label hapten, it is established that one threonine and one valine are very close to the paramagnetic hapten.

Reprinted from Biochemistry, 1984, 23, 1138.

Magnetic Resonance of a Monoclonal Anti-Spin-Label Antibody†

Jacob Anglister,‡ Tom Frey, and Harden M. McConnell*

ABSTRACT: The nuclear magnetic resonance spectra of monoclonal Fab antibody fragments have been recorded in the absence and presence of a specific spin-label dinitrophenyl hapten. The difference spectra reveal the presence of 11–12 aromatic amino acids in the region of the combining site. By selective deuteration of this hybridoma antibody, these amino acids have been identified as three tryptophans, six or seven tyrosines, one phenylalanine, and one histidine. Difference spectra have also been recorded that depend on ring-current chemical shifts. The latter difference spectra show that among the 11–12 amino acids there are two tyrosines and one tryptophan in close proximity to the dinitrophenyl (DNP) ring. On the basis of ultraviolet absorption measurements, it is concluded that this tryptophan is stacked against the DNP ring. Selective deuteration of hybridoma antibodies directed against a paramagnetic hapten provides a powerful new approach for the study of the structural basis of antibody diversity and specificity.

Antibody molecules play a crucial role in defense against infection. When confronted by almost any foreign molecule, the immune system is able to produce antibody proteins of high affinity and exquisite specificity. The structural basis of antibody specificity has been one of the major concerns of immunochemistry for many years. Statistical analysis of antibody sequences shows that this specificity is determined by relatively short segments, the hypervariable regions (Wu & Kabat, 1970). Crystallographic studies reveal that the combining sites are formed by these hypervariable regions [for recent reviews, see Amzel & Poljak (1979) and Pecht (1982)]. Most of the amino acids in the nonhypervariable region form a rigid, relatively invariant framework, termed the immunoglobulin fold. This framework consists of two layers of antiparallel β-pleated sheets held together by a disulfide bond and enclosing a hydrophobic interior.

Complexes formed between haptens and specifically elicited anti-hapten antibodies have not yet been investigated by X-ray crystallography or nuclear magnetic resonance (NMR)[1]

† From the Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305. *Received August 26, 1983.* This work was supported by NIH Grant 5R01 AI13587, NIH Grant RR07005, and NSF Grant PCM 8021993 and by NSF Grant GP23633 and NIH Grant RR00711 to the Stanford Magnetic Resonance Laboratory.

‡ Chaim Weizmann Fellow.

[1] Abbreviations: Fab, antigen binding fragment of antibody; SL, spin-label (see Materials and Methods for structure); DNP, dinitrophenyl; DNP-Gly, (2,4-dinitrophenyl)glycine; EDTA, ethylenediaminetetraacetic acid; NO, nitroxide; Fv, variable-region fragment of antibody; FabSL and FabDNP-Gly, complexes of antibody with indicated antigen; Tris, tris(hydroxymethyl)aminomethane. D(amino acid) means protein was grown with indicated amino acids deuterated; H(amino acid) means that the spectral features are mainly from the indicated amino acid.

0006-2960/84/0423-1138$01.50/0

Reprinted from Biochemistry, 1984, 23, 5372

Distances of Tyrosine Residues from a Spin-Label Hapten in the Combining Site of a Specific Monoclonal Antibody†

Jacob Anglister,‡ Tom Frey, and Harden M. McConnell*

ABSTRACT: The nuclear magnetic resonance spectra of an Fab fragment of a monoclonal antibody specifically directed against a nitroxide spin-label hapten have been recorded at different concentrations of the hapten. The hybridoma producing this antibody was grown on deuterated phenylalanine, tryptophan, and 3,5-dideuteriotyrosine or 2,6-dideuteriotyrosine. Difference spectra—without hapten minus with hapten—were calculated for each concentration of hapten. The difference spectra reveal five well-resolved singlet proton resonance signals from tyrosine deuterated in the 3,5-positions (H 2,6 Tyr) and nine from tyrosine deuterated in the 2,6-positions (H 3,5 Tyr). The measured intensities of these signals as a function of combining site occupation have been interpreted in terms of a theory involving intrinsic line widths (T_2), the hapten off-rate (k), and distances to the paramagnetic center. Good agreement with theory is found for all of the isolated proton signals. The best estimate of k is 350 s^{-1}; distances in the range 13 to <9 Å are calculated. Extension of this analysis to other amino acids is discussed.

Paramagnetic probes bound to a macromolecule broaden the nuclear resonance signals of nearby nuclei in a way that is related to the distance between the nuclei and the probe (Carrington & McLachlan, 1967; Sternlicht et al., 1965a,b; Jardetzky & Roberts, 1981). When the resonance lines corresponding to the nuclei studied are well resolved and the broadening can be measured, distances can be accurately determined. The distances are usually in the range 15–20 Å (Wien et al., 1973). For smaller distances the broadening is so large that the resonance signals cannot be detected. Campbell et al. (1975) suggested a method to measure these shorter distances by varying the fractional occupancy of binding sites for paramagnetic probes. Semiquantitative results were obtained by titrating lysozyme with Gd^{3+}.

In the present paper we give a detailed analysis of broadening effects, which enables accurate determinations of distances smaller than 15 Å. The method is used to determine distances of tyrosine residues from the combining site of a monoclonal antibody (ANO2) that is specifically directed against a spin-labeled hapten. Our study employs a hybridoma grown on selected deuterated amino acids, so as to produce monoclonal antibody with resonance signals that come from unique protons in single amino acids. This approach was used previously to determine which amino acids are in the combining site region of this AN02 antibody (Anglister et al., 1984).

Materials and Methods

The synthesis of the spin-label hapten has been described (Balakrishnan et al., 1982).

The origin, maintenance, and labeling of the AN02 cell line have been described previously (Anglister et al., 1984)). Fab fragments were prepared by standard procedures. The equilibrium stability constant for the binding of the spin-label hapten is 4×10^6 mol/L at room temperature in PBS.[1] The stability constant of DNP-Gly is 2×10^6 mol/L under the same conditions.

Amino acids L-tryptophan-2′,4′,5′,6′,7-d_5, L-(4-hydroxyphenyl-3,5-d_2)alanine (tyrosine), L-(4-hydroxyphenyl-2,6-d_2)alanine-2-d_1 (tyrosine), and L-phenyl-d_5-alanine-3,3-d_2 were purchased from MSD Isotopes.

NMR spectra were taken on a JEOL 500-MHz spectrometer. Free induction decays were collected in 800 data points after 60° pulses. Delays were 1 s when a sweep width of ±4000 Hz was used; 10000 scans were taken per sample. No smoothing of the spectra by exponential multiplication was used.

Theory

Theoretical Background: Difference Spectra. In our previous work we have used isotopic difference spectra to determine which aromatic amino acids are in the combining site region of AN02. In the present work we use difference spectra to obtain information on the distances of these amino acids from the combining site—specifically distances from the paramagnetic electron on the spin-label nitroxide group. Under conditions of fast hapten chemical exchange between the combining sites in a solution of Fab (f), the transverse relaxation time T_2 of an amino acid proton is given by the formula

$$\frac{1}{T_2} = \frac{f}{T_{2M}} + \frac{1-f}{T_{2N}} \tag{1}$$

Here f is the fraction of the time the combining site is occupied by the hapten. The transverse nuclear relaxation times T_{2M} and T_{2N} apply to the solutions Fab(1) and Fab(0), that is, solutions in which the combining site is always occuped or always empty, respectively. The relaxation rate T_{2M}^{-1} itself is a sum of two terms:

$$\frac{1}{T_{2M}} = \frac{1}{T_{2M'}} + \frac{1}{T_{2N}} \tag{2}$$

where $(T_{2M'})^{-1}$ is the enhancement of the nuclear relaxation rate due to the hapten. The Solomon–Bloembergen dipolar enhancement of the transferse relaxation due to the unpaired electron spin is

† From the Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305. Received March 19, 1984. This work was supported by ONR Contract N00014-83-K-0349, NIH Grant 5R01 AI13587 NSF Grant GP 23633, NIH Grant RR07005, and NIH Grant RR00711 to the Stanford Magnetic Resonance Laboratory.

‡ Chaim Weizmann Fellow.

[1] Abbreviations: Fab, antigen binding fragment of the antibody; DNP-Gly, (2,4-dinitrophenyl)glycine; PBS, phosphate-buffered saline, pH 7.2; H 2,6 Tyr, tyrosine deuterated in the 3,5-positions; H 3,5 Tyr, tyrosine deuterated in the 2,6-positions.

0006-2960/84/0423-5372$01.50/0

Reprinted from Biochemistry, 1984, 23, 6470.

Nonaromatic Amino Acids in the Combining Site Region of a Monoclonal Anti-Spin-Label Antibody†

Tom Frey, Jacob Anglister, and Harden M. McConnell*

ABSTRACT: The nuclear magnetic resonance spectra of monoclonal Fab antibody fragments have been recorded in the absence and presence of the specific spin-label dinitrophenyl hapten. The difference spectra reveal the presence of about 50 amino acids in the region of the combining site. By selective deuteration and by use of double difference spectra, all the resonances in the spectral region −1 to 1.5 ppm have been identified. We have found that in the combining site region there are four or five valines, certainly three and possibly five threonines, three or four leucines, two or three isoleucines, and six or seven alanines. Selective deuteration of methionine and lysine reveals one methionine and two lysines in the difference spectra. All of these amino acids are estimated to be within 17 Å of the paramagnetic hapten. By using difference spectra involving low fractional occupancy of the combining site with the spin-label hapten, it is established that one threonine and one valine are very close to the paramagnetic hapten.

Much progress has been made in accounting for the diversity and specificity of antibodies through studies of amino acid sequences and immunoglobulin genetics. X-ray crystallographic studies have shown that the hypervariable loops are responsible for binding site structure and that the more highly conserved sequences in the variable region are responsible for forming the immunoglobulin fold, the structural motif upon which all variable regions are built (Wu & Kabat, 1970; Amzel & Poljak, 1979). The number of antibody–hapten complexes studied by X-ray crystallography has been far too small to enable one to predict combining site structure and specificity from the amino acid sequence. It is with this and related problems in mind that we have undertaken a nuclear magnetic resonance study of a monoclonal antibody and its interactions with the parmagnetic hapten that it is directed against.

Previous workers (Dower & Dwek, 1979) have shown that it is possible to use the differences between the NMR spectra of the antibody and the antibody–hapten complex to get information about the structure of the binding site. We have extended this concept by employing the broadening effect of the spin-label hapten and biosynthetic incorporation of deuterated amino acids to identify the aromatic amino acid present in the combining site region of our monoclonal AN02 antibody (Anglister et al., 1984a). We have also shown that hapten exchange is fast enough that titration of the binding site (Campbell et al., 1975) allows the determination of proton–spin-label distances for well-resolved resonances in the difference spectra (Anglister et al., 1984b).

In the present work, we have used these approaches to study the more complicated spectral region due to the nonaromatic amino acids. The complexity of the spectrum in this region makes it necessary to use double difference spectra. In this type of analysis, the normal difference spectra (without hapten minus with hapten) are calculated for Fab's that differ only in the incorporation of one selected deuterated amino acid. The two difference spectra are then subtracted, leaving only the contribution of the selected amino acid in the double difference spectrum. We have assigned a large number of the resonances in the difference spectrum by this technique and in some favorable cases have made estimates of the distances between the amino acids and the paramagnetic hapten. It is hoped that such information, together with the (as yet unknown) amino acid sequence, will lead to a useful model of the combining site. Our present work and previous work on this problem clearly indicate that there are significant structural changes upon hapten binding.

Materials and Methods

The synthesis of the spin-label hapten has been described (Balakrishnan et al., 1982). The chemical formula can also be found in Anglister et al. (1984a).

The origin, maintenance, and labeling of the AN02 cell line have been described previously (Anglister et al., 1984a). Fab fragments were prepared by standard procedures. The amino acids perdeuterated L-lysine, L-threonine, and L-isoleucine were purchased from Cambridge Isotopes Laboratories. Perdeuterated L-alanine, L-leucine, L-valine, and L-[*methyl*-$^2H_3$]methionine were purchased from MSD Isotopes. Medium labeled with deuterated alanine included 3–4 mM alanine. In all other cases, the amino acids were in the regular quantities for RPMI medium. NMR spectra were taken with a JEOL 500-MHz spectrometer. The concentration of Fab was in the range (1.3–3) × 10^{-4} M, and sample volumes were 550 μL. Free induction decays were collected in 8000 data points after

† From the Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305. *Received May 16, 1984.* This work was supported by ONR Contract N00014-83-K-0349 and National Institutes of Health Grant 5R01 AI13587-08 to H.M.M. and by California Division of the American Cancer Society Senior Fellowship S2-84 to J.A.

0006-2960/84/0423-6470$01.50/0

NMR technique for assessing contributions of heavy and light chains to an antibody combining site

Jacob Anglister, Tom Frey & Harden M. McConnell

Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305, USA

Nuclear magnetic resonance (NMR) has been used extensively to study the structure of antibody combining sites[1,2]. In recent studies we have observed the proton resonance spectra of the Fab fragment of a monoclonal anti-spin-label antibody derived from a hybridoma grown on various specifically deuterated amino acids[3,4]. The broadening of the proton resonance signals by the paramagnetic hapten, together with selective deuteration, has allowed the identification of most of the amino acids in the combining-site region of this antibody and has also provided estimates of distances between amino-acid protons and the unpaired electron[5]. Here we show how recombination of specifically deuterated heavy and light chains permits the assignment of single amino-acid proton resonance signals to either the heavy or light chain. In addition, the spectra of such recombinants demonstrate that their combining-site structures must be almost identical to the native structure.

The murine IgG_1 monoclonal anti-spin-label antibody (ANO2) was derived from a hybridoma grown on specifically deuterated amino acids, as described previously[3]. Two preparations of Fab were obtained: one (Fab I) was obtained from the hybridoma grown on perdeuterated tryptophan, perdeuterated phenylalanine and perdeuterated tyrosine; the second (Fab II) was obtained from the hybridoma grown on perdeuterated tryptophan, perdeuterated phenylalanine, and tyrosine deuterated at the 2,6 ring positions. The Fab fragments, prepared as described previously[3], were partially reduced and alkylated. The heavy (H)-chain fragment and the light (L) chain were separated on a DEAE column under denaturing conditions (8 M urea, Tris-glycine buffer, *p*H 8.2), then the heavy-chain fragments and light chains were recombined to produce the recombinants H(I)L(II) and H(II)L(I). The urea solution was made 1 M in propionic acid. The recombinants were dialysed against 1 M propionic acid and then twice against each of the following: water, 5 mM acetate buffer *p*H 5.8, 5 mM acetate buffer *p*H 5.8 containing 0.15 M NaCl, and phosphate-buffered saline *p*H 7.2 (at least 12 h for each). A similar procedure for reconstitution of a whole antibody molecule has been described previously by several groups[6,7].

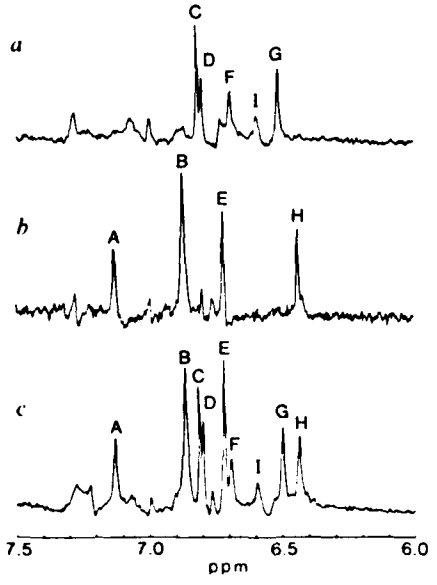
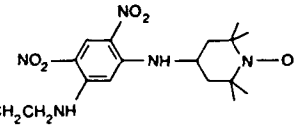


Fig. 1 NMR difference spectra for selectively deuterated Fab fragments of a monoclonal anti-spin-label antibody. *c*, Signals A–I are each due to pairs of protons in the 3,5 ring positions of tyrosine residues in the combining-site region of the ANO2 antibody. Spectra *a* and *b* give the corresponding signals from protons in the light chain and heavy chain, respectively. Note that the spectrum in *c* is, to a good approximation, the sum of the spectra in *a* and *b*. (See text for details.)

Figure 1 shows the proton resonance difference spectra ΔH(II)L(II), ΔH(II)L(I) and ΔH(I)L(II), where Δ = proton resonance spectrum of Fab − proton resonance spectrum of the Fab fragment with paramagnetic spin-label hapten bound. The paramagnetic spin-label hapten has the formula



The proton resonance signals in Fig. 1*a* arise from the 3, 5 protons of tyrosines in the light chain that are in the combining-site region (within 17 Å of the unpaired spin[5]). The signals in Fig. 1*b* are due to the 3,5 protons of the tyrosines of the heavy chain in the combining-site region.

That the spectrum in Fig. 1*c* is very nearly the sum of the spectra in Fig. 1*a* and *b* represents compelling evidence that the combining-site structures are virtually the same in all three cases; this is because resonance positions, intensities and linewidths are sensitive to conformation, motional freedom and environment. A similarity in structure between native and reconstituted IgG was observed previously using circular dichroism and optical rotatory dispersion[7,8].

Our spectra show that there are five tyrosines in each chain in the combining-site region. Signal I from the light chain is weaker because it is farther from the unpaired electron. In each tyrosine signal the two proton resonance lines are equivalent due to rapid rotation of the aromatic ring. Similar conclusions are reached from the proton resonance spectra of the Fab fragment containing tyrosine deuterated in the 3,5 positions (data not shown), that is, there are five tyrosines in each chain, and the conformation of the combining site in this reconstituted Fab is very similar to the conformation in the native Fab.

Signal B in Fig. 1*b* arises from two tyrosines, as is evident from the observed intensity. Figure 2 shows that this signal is split in a difference spectrum, Δ = spectrum of Fab containing the reduced spin label (nitroxide to hydroxylamine) − resonance spectrum of Fab to which the paramagnetic spin label is bound. Interestingly, all the tyrosine proton resonance signals in Fig. 2 remain sharp, and some shift in resonance position when the non-paramagnetic hapten is in the combining site. Presumably, some of these tyrosine residues are in contact and/or are hydrogen-bonded to the hapten. Nonetheless, the motional freedom remains large enough to yield sharp resonance signals (4–7 Hz linewidth at half height).

In previous studies using double difference spectra, we demonstrated the presence of three to five threonines in the combining-site region[4]. In the present study we have used double difference spectra involving two recombinant Fab fragments to establish the presence of two threonine residues on the light chain and two to three threonine residues on the heavy chain in the combining-site region. One Fab fragment was not labelled and the other had deuterated threonine in the light chain (data not shown).

Note that the 'combining-site region' is defined as the region within 17 Å of the unpaired electron, and that proton resonance signals in this region are obtained from the difference spectra Δ = proton resonance spectrum of Fab − proton resonance spectrum of Fab with paramagnetic spin-label hapten bound. The geometry of the hapten is such that the protons of all amino acids in contact with the hapten are included in this combining-

DIVERSITY OF MOLECULAR RECOGNITION: THE COMBINING SITES OF MONOCLONAL ANTI SPIN LABEL ANTIBODIES

Harden M. McConnell, Tom Frey, Jacob Anglister, Mei Whittaker

Stauffer Laboratory for Physical Chemistry
Stanford University
Stanford, CA 94305

INTRODUCTION

Physical chemistry includes the study of how atoms come together to form molecules, and how combinations of molecules can interact with one another to form aggregates, crystals, and macromolecules. One of the most challenging problems in the area of macromolecules is the problem of protein structure, the problem of finding the "code" that specifies how a given amino acid sequence gives rise to a three-dimensional protein structure, such as an enzyme, with a highly specific biochemical function. This problem has been already attacked with considerable success using NMR methods, through studies of the folding-unfolding of polypeptides and proteins.[1] Another important facet of the problem of protein structure, and the evolution of protein structures, concerns the manner in which amino acid sequences corresponding to exons, are assembled as structural units ("modules") to form three-dimensional structures with specific functions.[2]

The purpose of the present article is to indicate that one can use NMR to study the structure of the combining sites of monoclonal antibodies. These combining site structures involve both the protein folding problem, the "module assembly problem," and the molecular complementarity problem--the "fit" of hapten/antigen to the combining site. Antibodies constitute a unique class of proteins for studying these problems.

Our NMR technique for studying the structure of antibody combining sites employs an intrinsic probe. We use a paramagnetic spin label hapten to facilitate the identification of the amino acids in combining sites, and to obtain structural information based on hapten-amino acid distances. As discussed later, our method in essence provides a "fingerprint" of the antibody combining site region, this fingerprint depending in a sensitive way on both the amino acid composition and structure of the combining site. Our paramagnetic spin label hapten is the natural target for the antibody combining site, since the antibody is derived from the cells of mice immunized with the hapten. Thus, there is no issue as to whether our paramagnetic nitroxide group "perturbs" the native structure of the protein. A second unique feature of antibodies is their diversity--not only can antibodies be formed against almost any substance, but typically, many different antibodies can be formed that bind to one single substance--in our case, the paramagnetic hapten. A particularly relevant study of

NMR technique for assessing contributions of heavy and light chains to an antibody combining site

Jacob Anglister, Tom Frey & Harden M. McConnell

Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305, USA

Nuclear magnetic resonance (NMR) has been used extensively to study the structure of antibody combining sites[1,2]. In recent studies we have observed the proton resonance spectra of the Fab fragment of a monoclonal anti-spin-label antibody derived from a hybridoma grown on various specifically deuterated amino acids[3,4]. The broadening of the proton resonance signals by the paramagnetic hapten, together with selective deuteration, has allowed the identification of most of the amino acids in the combining-site region of this antibody and has also provided estimates of distances between amino-acid protons and the unpaired electron[5]. Here we show how recombination of specifically deuterated heavy and light chains permits the assignment of single amino-acid proton resonance signals to either the heavy or light chain. In addition, the spectra of such recombinants demonstrate that their combining-site structures must be almost identical to the native structure.

The murine IgG_1 monoclonal anti-spin-label antibody (ANO2) was derived from a hybridoma grown on specifically deuterated amino acids, as described previously[3]. Two preparations of Fab were obtained: one (Fab I) was obtained from the hybridoma grown on perdeuterated tryptophan, perdeuterated phenylalanine and perdeuterated tyrosine; the second (Fab II) was obtained from the hybridoma grown on perdeuterated tryptophan, perdeuterated phenylalanine, and tyrosine deuterated at the 2,6 ring positions. The Fab fragments, prepared as described previously[3], were partially reduced and alkylated. The heavy (H)-chain fragment and the light (L) chain were separated on a DEAE column under denaturing conditions (8 M urea, Tris-glycine buffer, *p*H 8.2), then the heavy-chain fragments and light chains were recombined to produce the recombinants H(I)L(II) and H(II)L(I). The urea solution was made 1 M in propionic acid. The recombinants were dialysed against 1 M propionic acid and then twice against each of the following: water, 5 mM acetate buffer *p*H 5.8, 5 mM acetate buffer *p*H 5.8 containing 0.15 M NaCl, and phosphate-buffered saline *p*H 7.2 (at least 12 h for each). A similar procedure for reconstitution of a whole antibody molecule has been described previously by several groups[6,7].

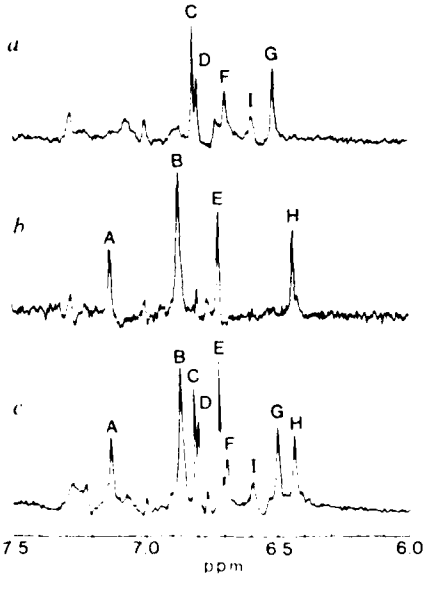
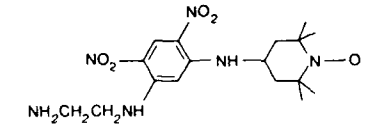


Fig. 1 NMR difference spectra for selectively deuterated Fab fragments of a monoclonal anti-spin-label antibody. *c*, Signals A–I are each due to pairs of protons in the 3,5 ring positions of tyrosine residues in the combining-site region of the ANO2 antibody. Spectra *a* and *b* give the corresponding signals from protons in the light chain and heavy chain, respectively. Note that the spectrum in *c* is, to a good approximation, the sum of the spectra in *a* and *b*. (See text for details.)

Figure 1 shows the proton resonance difference spectra ΔH(II)L(II), ΔH(II)L(I) and ΔH(I)L(II), where Δ = proton resonance spectrum of Fab − proton resonance spectrum of the Fab fragment with paramagnetic spin-label hapten bound. The paramagnetic spin-label hapten has the formula



The proton resonance signals in Fig. 1*a* arise from the 3, 5 protons of tyrosines in the light chain that are in the combining-site region (within 17 Å of the unpaired spin[5]). The signals in Fig. 1*b* are due to the 3,5 protons of the tyrosines of the heavy chain in the combining-site region.

That the spectrum in Fig. 1*c* is very nearly the sum of the spectra in Fig. 1*a* and *b* represents compelling evidence that the combining-site structures are virtually the same in all three cases; this is because resonance positions, intensities and linewidths are sensitive to conformation, motional freedom and environment. A similarity in structure between native and reconstituted IgG was observed previously using circular dichroism and optical rotatory dispersion[7,8].

Our spectra show that there are five tyrosines in each chain in the combining-site region. Signal I from the light chain is weaker because it is farther from the unpaired electron. In each tyrosine signal the two proton resonance lines are equivalent due to rapid rotation of the aromatic ring. Similar conclusions are reached from the proton resonance spectra of the Fab fragment containing tyrosine deuterated in the 3,5 positions (data not shown), that is, there are five tyrosines in each chain, and the conformation of the combining site in this reconstituted Fab is very similar to the conformation in the native Fab.

Signal B in Fig. 1*b* arises from two tyrosines, as is evident from the observed intensity. Figure 2 shows that this signal is split in a difference spectrum, Δ = spectrum of Fab containing the reduced spin label (nitroxide to hydroxylamine) − resonance spectrum of Fab to which the paramagnetic spin label is bound. Interestingly, all the tyrosine proton resonance signals in Fig. 2 remain sharp, and some shift in resonance position when the non-paramagnetic hapten is in the combining site. Presumably, some of these tyrosine residues are in contact and/or are hydrogen-bonded to the hapten. Nonetheless, the motional freedom remains large enough to yield sharp resonance signals (4–7 Hz linewidth at half height).

In previous studies using double difference spectra, we demonstrated the presence of three to five threonines in the combining-site region[4]. In the present study we have used double difference spectra involving two recombinant Fab fragments to establish the presence of two threonine residues on the light chain and two to three threonine residues on the heavy chain in the combining-site region. One Fab fragment was not labelled and the other had deuterated threonine in the light chain (data not shown).

Note that the 'combining-site region' is defined as the region within 17 Å of the unpaired electron, and that proton resonance signals in this region are obtained from the difference spectra Δ = proton resonance spectrum of Fab − proton resonance spectrum of Fab with paramagnetic spin-label hapten bound. The geometry of the hapten is such that the protons of all amino acids in contact with the hapten are included in this combining-

END

DTIC

6-86